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13. ABSTRACT ***(Maximum 200 Words)***

Epidermal growth factor (EGF) receptor is expressed in many normal tissues, including mammary epithelium. Overexpression or mutation of the EGF receptor causes neoplastic transformation in many cell types. Several studies have implicated the EGF receptor as a good prognostic marker in breast cancer, but its role in the etiology and progression of this malignancy is still under dispute. We proposed to utilize a genetic approach to investigate this tissue by developing transgenic mice in which the EGF receptor gene in the mammary gland will be inactivated at the onset of the first lactation. The construct of the targeting was made and was introduced into the ES cells. Positive clones were injected to generate chimeric mice, which give rise to germ line transmission. After several mating and crossing, we have generated our mouse model. Now we have started the treatment and analysis. We hope very soon that we will know the answer whether the EGF receptor plays a functional role in the etiology and progression of mammary carcinoma. The answer to this question would indicate the feasibility of using the EGF receptor as a target in breast cancer therapy. We are also going to extend our studies by crossing our mouse model to Akt1 knockout mice we have recently generated.

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Introduction

Breast cancer has been the leading cause of death among non-smoking women and thus has been the focus of intensive research. For the last decade or so, many researchers have concentrated on understanding the molecular basis of breast cancer. Since the epithelial cells of the breast are regulated by a variety of hormones and growth factors, it appears that abnormal hormonal milieu might be one of the critical factors in the development of breast cancer. EGF (epidermal growth factor) is a growth-stimulating factors and acts as an autocrine and paracrine growth factor. The expression of both EGF and is positively regulated by estrogen and progesterone receptors [1].

The EGF receptor is a 170 kDa transmembrane glycoprotein, which belong to the tyrosine kinase receptor family. The role of the EGF receptor in breast cancer has been studied in great detail over the last decade [2], but it still remains under debate. The expression of EGF receptors on normal breast epithelial cells are low and elevated expression of EGF receptors occurs in about 40% of primary breast cancers. There is a clear reverse relationship between EGF receptor expression and estrogen receptor expression, as well as between the expression of EGF receptor and progesterone receptor [3]. High levels of EGF receptor are associated with poor tumor differentiation, high tumor grade, aneuploidy and high rate of cell proliferation [4]. There is still no consensus, however, on the significance of EGF receptor in breast cancer prognosis or its correlation with the relapse-free survival and overall survival [5,6]. In node-positive patients, the EGF receptor appears to be a good prognostic marker, but in the node-negative patients, the EGF receptor does not appear to have a prognostic value [7,8].

The development of the transgenic mouse and gene knockout techniques provides an exceptional opportunity to elucidate the role of the EGF receptor signal pathway in mammary gland cancer [9]. Three groups have generated null mutations of the EGF receptor in mouse by homologous recombination. The phenotype of the null mutation in mouse turned out to be different in different genetic backgrounds. However, no female mice live long enough to develop mature mammary gland [10-12]. Thus, the null mutation of the EGF receptor in mouse does not provide us with a model to study the role of the EGF receptor in mammary cancer. Therefore, we need to generate a mutant mouse lacking EGF receptor specifically in the mammary gland. Recent success of cre/loxP mediated gene knockout technique [13], which allows one to change the gene of interest in a tissue-specific manner, provides us with the tool to generate mice lacking EGF receptor only in the mammary gland. This mutant mouse will be our model to study the role of the EGF receptor in mammary cancer. We will evaluate the effect of the EGF receptor on the incidence of mammary tumors induced by chemical carcinogenesis. Analysis of this mutant mouse should reveal the role of the EGF receptor in the etiology and progression of breast cancer and will indicate the significance of the EGF receptor as a therapeutic target.

Recent studies have shown that Akt gene is amplified in some breast cancer patients [14]. Akt, also known as protein kinase B (PKB), encodes for a serine/threonine kinase, which contains three members Akt1(PKBα),

Akt2(PKBβ) and Akt3(PKBγ). Akt1 and Akt2 are expressed ubiquitously, while Akt3 is expressed highly in brain and testis. Those three isoforms display a greater than 80% identity. Akt is regulated by a wide range of growth factor, such as EGF. The activation of Akt results in several biological effects, including cell survival. Amplification and overexpression of Akt has been observed in many types of cancer, including breast cancer [14]. We are generating all three isoforms of Akt knockout mice. We have just generated the Akt1 knockout mice that are normal and fertile except a little smaller than the wild type littermates. We are going to cross the Akt1 knockout mice with our mouse lacking EGF receptor in mammary gland to generate a mouse strain that lacks Akt1 as well as EGF receptor in the mammary gland. This mutant strain of mouse should reveal the role of both EGF receptor and Akt1 in breast cancer.

Revision for the Final Report

Body (revised)

1) **Construction of the targeting vector for EGF receptor gene.** (Task 1 in statement of work)

The targeting vector was finished at the first year as show in figure 1.

2) **Cloning of the promoter region of WAP (white acid protein) gene and construction of WAP-*cre*.** (Task 2 in statement of work)

We have cloned the promoter region of WAP and made the construct containing 3.5 kb WAP promoter and *cre* gene. After we made the construct, Jackson Lab has obtained the license from DuPont to sale transgenic mouse containing *cre* gene, including WAP-*cre* mouse, which is the same one that we plan to generate. Therefore, in stead of generating our own strain of mouse, we have asked Dr. Wagner at NIH to provide us the WAP-*cre* mice and he has been very kind to send us the mice. Now the WAP-*cre* mouse should be available at Jackson Lab.

3) **Electroporation of ES cells with EGF receptor construct.** (Task 3 in statement of work)

We have introduced the EGF receptor targeting vector we constructed into ES cells by electroporation. After ten days of G418 selection, we have isolated about 500 colonies. Each individual clone was expanded and the DNA from each clone is isolated for Southern blot analysis. The probe we used for Southern blot analysis is a ~500 bp fragment 5'-end outside targeting construct (see figure1). After analyzing more than 400 individual clones, we identified 11 positive ones. We expanded the ES cells from those 11 positive clones for more DNA to confirm those ES cells had gone through the correct DNA recombination by Southern blot analysis. We also use the *neo* gene as a probe to determine a single integration. (This work was finished the second year.)

4) **Electroporation of ES cells with the *cre* gene to remove the *neo* gene cassette.** (Task 3 in statement of work)

One of positive clones was selected to remove the *neo* gene cassette in ES cells. The ES cells were electroporated with a plasmid containing *cre* gene under the control of CMV promoter. About 20 G418 negative clones were picked up. However, Southern blot analysis revealed that all of them were deletion including exon 1 and the *neo* cassette, and we expected to get 50% exon 1 and the *neo* cassette deletion and 50% only *neo* cassette deletion. We further tried several times with the same result. After attending the "Mouse Molecular Genetics" meeting, we found out some other labs have the same problem as we have. In order to solve the problem, we took several approaches. We used a different promoter, such as PGK, to express *cre* gene in ES cells and lowered the amount of the *cre* gene construct for eletroporation. After several trials, we were able to obtain ES cells with only *neo* cassette deletion. At the meeting, we have also learned that we can have both alleles with *neo* cassette deletion for our study to understand the role of EGF

receptor in mammary gland tumor. Recently, many labs have shown transgenic mice containing *cre* recombinase is sufficient to delete both alleles of the exons flanking with loxP sites for more than 80% efficiency. For our studies, more than 50% deletion of EGF receptor gene in mammary gland will be sufficient enough. Therefore, it becomes not necessary for us to generate both exon 1 and the *neo* cassette deletion mice. We will only need to generate mice that carrying two copies of loxP sites flanking exon 1 of EGF receptor. As a result, it will save a lot of time to generate our mouse model for our studies.

5) **Generating mouse containing loxP sites flanking exon 1 of EGF receptor gene.** (Task 5 and 6 in statement of work)

To generate mouse containing loxP sites flanking exon 1 of EGF receptor, we injected ES cells containing exon 1 flanking loxP sites to generate chimeric mice. However, it appears that none of the chimeric animals are able to give rise to germ line transmission. Recent studies from Westphal's Lab and others have shown that transgenic mice carrying a *cre* gene under the control of EIIa promoter are able to delete sequences at the zygote sage as well as to delete the *neo* gene cassette at a very high percentage [13]. Therefore, we also injected the ES cells that contain the targeting vector (figure 1) to generate chimeric mice. Those chimeric animals were able to give rise to germ line transmission. The heterozygous mice that contain the targeting vector were crossed with a transgenic mouse carrying a *cre* gene under the control of EIIa promoter, which was kindly provided by Dr. Westphal. In order to use PCR to genotype the mice instead of Southern blotting, we design two sets of oligo primers that flanking the sequences of two loxP sites between 100 bp to 200 bp apart (see figure 1, primer 1/primer 2 and primer 3/primer 4), so that we are able to use PCR to determine whether the mice is carrying a specific loxP site in the genome as well as carrying a deletion of the *neo* gene cassette. For primer 1/primer 2, the mutant allele has insertion of a loxP site of ~50 bp. Therefore, the PCR product will show two bands when both the wild type and mutant alleles exist. For primer 3/primer 4, the PCR product will only show two bands when the *neo* gene cassette is deleted. The lower band is the wild type allele, and the upper one is the mutant allele that contains one loxP site without the *neo* gene cassette. In the case that the *neo* gene cassette is not deleted, the PCR product will only show one band (the wild type allele) because the other band would have been more than 2.5 kb, which will be over the limit of Taq polymerase to produce. We have used this method to genotype our mice from their DNA very efficiently. Crossing with EIIa-*cre* mice, the *neo* gene cassette from two different lines of mice that are carrying the targeting vector is deleted (see table 1). The percentage of deletion of the *neo* gene cassette was very high. Those mice with deletion of the *neo* gene cassette are further crossing with the wild type mice for germ line transmission as well as to mate out the EIIa-*cre* transgene from the genome. Therefore, no further deletion between the loxP sites will occur. More than 50% of the mice are able to give rise to germ line (see table 1). Mating those mice with loxP sites flanking exon1 to homozygotes as well as with WAP-*cre* mice provided by Dr. Wagner were able to generate our mouse model to study the role of EGF receptor in mammary gland tumorigensis. (Most of the work was finished last year.)

6) **Analysis and treatment of the mouse model** (task 7-9). (revised)

We believe that we have generated our mouse model to study the role EGF receptor in mammary gland tumor. We have started to induce mammary gland tumor for our mouse model with DMBA (7,12-dimethylbensanthracene) treatment, but we do not have any data at this time to report. We are also going to exam our model mice before the deletion of exon 1. This is an important part of the studies, because we have only hypothesized that our mice model will be normal with loxP insertion, but we have not proven it yet. Recently I attended the Mouse Molecular Genetics Meeting at Cold Spring Harbor; there were several reports of abnormal phenotype with the loxP sites inserted in the genome alone. Therefore, we need to analyze the phenotype of those mice with only insertion of the loxP sites. At the same time, we are going to determine the percentage of the deletion of exon 1 EGF receptor gene by Southern blot analysis, which is also important for our studies. Meanwhile, we will extend our study with crossing our EGF receptor mouse model to Akt1 knockout mouse, which we have recently generated (not published), to determine the role of both EGF receptor and Akt1 in tumorigensis. The Akt gene has been reported to be amplified in some breast cancer patients. We are also generating both the Akt2 and Akt3 knockout mice. In the future, we will cross them to our EGF receptor mouse model with the Akt2 and Akt3 knockout mice as well. Since we have problem to generate to ES cells without *neo* cassette and changed to the new method to delete the *neo* cassette in mouse, we started this task 7-9 much later then we should. It will take for a while to finish this project.

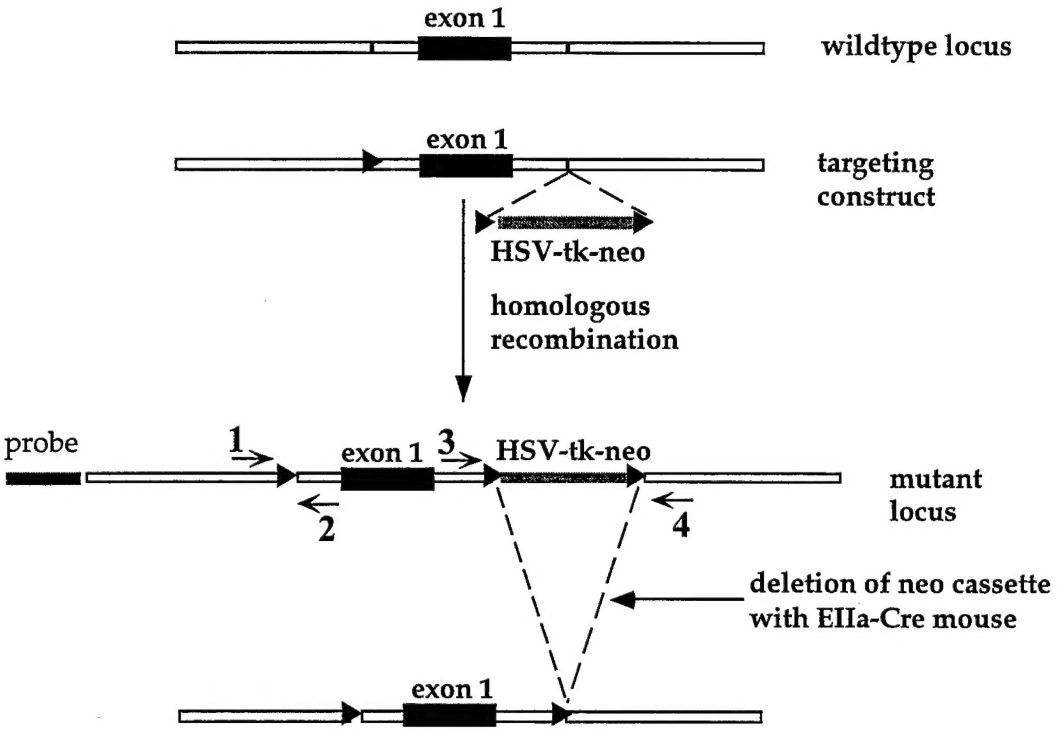

Fig 1. Strategy to generate flanking by loxP sites in ES cells.
Genetic locus, targeting construct and mutant locus as well as Cre-mediated deletion of neo cassette are shown.
The loxP site: ▶. Southern probe: ▬ . PCR primers: 1,2,3,4.

Table 1. Deletion of *neo* cassette by crossing with EIIa-Cre mice.

CLONE	*% DELETION*	*% GERM LINE*
EGFR-137	89% (8/9)	50% (4/8)
EGFR-229	60% (6/10)	67% (4/6)

Key Research Accomplishments

1. Chimeric mice that gave rise to germ-line transmission.
2. Mice carrying the targeting vector.
3. Mice carrying two loxP sites flanking exon 1 of EGF receptor gene with the deletion of the *neo* gene cassette by mating with EIIa-Cre transgenic mice.
4. Mice carrying the genotype of $EGFR^{flox/+}$ by mating out the EIIa-*cre* transgene from the mouse genome.
5. Mice carrying the genotype of *cre*$EGFR^{flox/+}$ by mating with WAP-*cre* transgenic mice.
6. Mice carrying the genotype of *cre*$EGFR^{flox/flox}$.

Reportable Outcomes

1) Two abstracts for two meetings (Abstract see Appendices).
2) Develop a mouse model in which the EGF receptor gene in the mammary gland will be inactivated at the onset of the first lactation. During the period of generating our mouse model, we have used the EIIa-*cre* mice to delete the *neo* gene cassette in the EGF receptor successfully. From the two lines of mice we used to cross with EIIa-*cre* mice, there is one line that we have eight out of nine (89%) mice with the deletion of the neo gene cassette and among the eight mice, four gave rise to germ line transmission (50%). We have similar situation with the other line, six out of ten (60%) mice with the deletion and among the six, four germ line transmission (67%). From our studies, it has shown that EIIa-*cre* mouse is able to delete the *neo* cassette efficiently (see table 1). Using the EIIa–*cre* mouse to delete the *neo* gene cassette has several advantages:
   1. One less step of ES cell manipulation. Since there is no need to delete the neo cassette in the ES cells, it leads to no further manipulation of ES cells. The less you have to manipulate the ES cells, the higher changes the ES cells will give rise to germ line transmission. For us, it appears that we are not able to get germ line transmission from ES cells that have gone through two rounds of manipulation, but are able to get germ line with ES cells that have gone through one.
   2. High efficiency. We have achieved between 60%-89% of the deletion of the *neo* gene cassette and 50% to 67% of the mice with deletion are able to give rise of germ line transmission. The reason that not all the mice have the deletion from tail tip DNA could be explained as the deletion occurs after the zygote stage, and that is why no deletion can be detected for some mice and some mice with the deletion are not able to give rise to germ line transmission.
   3. Easy. It is very easy to delete the neo gene cassette. You only need to cross the mice with EIIa-*cre* mice.
   4. No need to generate the EIIa-*cre* mouse. EIIa-*cre* mouse is now available at Jackson Lab.
   5. The disadvantage of using EIIa-*cre* mouse is that it takes longer time to generate mice with the deletion of *neo* cassette compared to delete the *neo*

gene cassette in ES cells, since two crosses are required to delete the *neo* gene cassette and to determine germ line transmission as well as to mate out the EIIa-*cre* transgene.

Conclusions (revised)

This has been a long time project. We believe that we have finished the first part to generate our mice model, but not the second part of treatment and analysis of our mouse model. However, it was a very important grant for me to start my career in breast cancer research. I was able to use this grant to start setting up the cre/loxP conditional knockout mice when very few scientists have used this technique. Although we have not finished the project on time, we have generated our mouse model for this studies and we will continue these important studies. We are also going to extend our studies with this mouse model with our Akt1 knockout mice as well as with Akt2 and Akt3 knockout mice in the future. I hope the Army will continue funding basic science research of breast cancer, so that in the near future we will be able to cure the disease of breast cancer.

The reasons for not completing this project on time are due to several factors. One of them would be the problem we had during deleting the *neo* cassette in the ES cells but also maintaining the ability of the ES cells to give rise to germline transmission. We used another method to solve the problem by generating mice with the *neo* cassette but deleting the *neo* cassette by crossing to EIIa-Cre mice. However, we had wasted a lot of time trying to delete the *neo* cassette in ES cells. The other reason will be that the new method of deletion of *neo* cassette in mouse has to add two extra crosses with mice, and each cross takes about 4-6 months, which we did not include in the original project. Therefore, we are not able to finish the project on time.

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Appendices

1. Publication and meeting abstract

a). Trans-Tech Meeting 2000 (May 17-20, 2000)

Title: **AN EASY AND SIMPLE WAY TO REMOVE THE *NEO* CASSETTE FROM THE MOUSE GENOME**

Abstract: Using Cre-loxP system for conditional knockout, there is one step that requires to delete the *neo* cassette from the loxP-loxP-neo-loxP in the mouse genome. The *neo* cassette was used as a positive selection marker, but it has been reported that leaving it in the genome exhibited as a null mutation by blocking the normal splicing. Here we report an efficient and novel way to excise only the neo cassette but leaving the third loxP site untouched, without manipulating the ES cells or generating a new strain of mouse. We used this method for the conditional knockout for EGF receptor gene. The targeting vector contains exon 1 of EGF receptor gene flanked by two loxP sites with a *neo* cassette also flanked by a loxP site (loxP-loxP-neo-loxP). The targeting vector was introduced into the ES cells by electroporation, and the heterozygous mouse containing the targeting vector was crossed with EIIa-Cre mouse. Our studies of the offspring from this mating show that we have deleted the *neo* gene cassette successfully.

b). Era of Hope 2000 (June 8-12, 2000)

Title: **TRANSGENIC MOUSE MODEL TO STUDY THE ROLE OF EGF RECEPTOR IN BREAST CANCER**

Abstract: Epidermal growth factor (EGF) receptor is expressed in many normal tissues, including mammary epithelium. Overexpression or mutation of the EGF receptor causes neoplastic transformation in many cell types. Although the role of EGF receptor in breast cancer has been studied in great detail over the last decade, it still remains under debate.

To investigate the role EGF receptor in breast cancer, we propose to utilize a genetic approach, by developing transgenic mice in which the EGF receptor gene only in the mammary gland will be inactivated at the onset of the first lactation. That is because the null mutation of EGF receptor mouse does not live long enough to adult.

The mutant mouse model to study the role of EGF receptor in mammary cancer will carry loxP sequences insertions flanking exon 1 of EGF receptor genes as well as *cre* gene under the control of mammary-specific WAP (white acid protein) promoter which will delete the EGF receptor gene only in the mammary gland. In order to achieve it, the targeting vector contains exon 1 of EGF receptor gene flanked by two loxP sequences with a neo cassette also flanked by loxP sequences. The targeting vector was introduced into the ES cells by electroporation. Positive clones with the correct recombination by Southern blot analysis were injected into ES cells. The heterozygous mouse crosses with EIIa-Cre mouse to delete the *neo* gene cassette. The next cross is to eliminate the EIIa–Cre from the genome. The following cross with WAP-Cre mouse is to introduce the cre gene. The mating between the last cross is to generate our mouse model to study EGF receptor in mammary gland.

We are now inducing mammary gland tumor of our mouse model with DMBA (7,12-dimethylbensanthracene). Analysis of mammary carcinogenesis in such mouse should reveal if EGF receptor plays a functional role in the etiology and progression of mammary carcinoma. The answer to this question would indicate the feasibility of using the EGF receptor as a target in breast cancer therapy.

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